

## Chapter Twelve

### Traditional and Novel Approaches to the Analysis of Plant Phospholipids

#### Introduction

Due to the importance of phosphorus analysis in the quantitation of phosphatides, this chapter begins with a detailed survey of wet chemical and instrumental methods of phosphorus analysis. Many of these analytical procedures have not yet been evaluated for routine analyses. This chapter includes a discussion of enzymatic analysis of phosphatides. The value of such procedures will depend on the availability of commercial enzyme kits such as one now available from Japan, and also on the broadening of the scope of such analysis to include not only the choline-containing species but other phosphatides as well. The chapter's final section covers the analysis of the phosphatides' subclasses, including their isolation from tissue, their chromatographic separation, identification and quantitation, and the determination of their individual molecular species.

#### Analysis of Phosphorus Content

Traditionally, phosphatides have been quantitated by analysis of their phosphorus content. As most of the material removed from crude oil by degumming is phosphatide, the residual phosphorus content of refined oil is a good indicator of the extent of degumming. Degumming of soy oil, for example, can reduce its phosphorus content from as high as 1000 ppm to 3–9 ppm (1). Maximum allowable phosphorus content in degummed soy oil is 250 ppm, according to the trading rules of the National Soybean Processors' Association (2).

For nearly 60 years, phosphorus analysis has been accomplished by one variation or other of the wet chemical analysis of Fiske and SubbaRow (3), in which molybdenum blue from molybdate, orthophosphate, and a reducing agent is measured colorimetrically. In 1947, Raymond Reiser wrote (4), "The results of phospholipid determinations in plant materials are as numerous as the authors, all of whom use a different procedure." The 1970s saw the development of instrumental methods, some of which expanded upon the wet chemical ones by automation, others of which followed completely different approaches. Some of these new methods discussed below already are in use, whereas others are presented to show the line of thought of researchers who still strive for innovation in phosphorus analysis. When the analyst considers a method, he must know its sensitivity, accuracy, and repeatability. He also must balance its convenience with its expense.

#### **Molybdenum blue and molybdovanadophosphate yellow.**

The various methods for complexing inorganic phosphate with molybdate all involve three routines. Initially, organic material must be digested and phosphorus converted to orthophosphate. Then color is developed, usually under controlled pH. Finally, a colorimetric determination is run, either on the same aqueous solution, or on an organic extract containing the colored species.

*Digestion to Inorganic Phosphate.* The digestion process, which eliminates all organic material, recently has been the object of two critical studies. Yee (5,6), who worked with amniotic and serum lipids, compared five acid digestions, all run at 225 C for 30 min: (a) perchloric acid; (b) perchloric acid with molybdate; (c) sulfuric acid/hydrogen peroxide/urea; (d) sulfuric acid/perchloric acid; and (e) sulfuric acid/perchloric acid/vanadium pentoxide. He concluded that one was as good as another, although digestion "e" worked best on sphingolipids. The analyst who wishes to mineralize a large oil matrix, however, must be warned that perchloric acid alone is unsuitable; most frequently, the perchloric acid/oil mixture will explode on heating. Acid digestion was not highly regarded, however, in the other comparative study. Daun and coworkers (7) applied four highly different digestion techniques to canola oil: (a) sulfuric acid/perchloric acid—the method of Rouser and coworkers (8), which gave poor reproducibility; (b) saponification—the technique of Hartman and coworkers (9), in which saponification by ethanolic sodium hydroxide takes place in an oven in a Teflon crucible and is followed by acidification and partition between hexane and water. This also gave poorly reproducible results and was labor-intensive; (c) the AOCS official Method Ca 12-55 (10)—a method that involves charring and ashing in the

presence of zinc oxide, then acidification to dissolve the ash, neutralization, and reacidification. This was "lengthy and cumbersome," taking two days to complete the ashing; and (d) the preferred method, the oxygen bomb technique (11)—ashing in a bomb with zinc oxide, water and oxygen, followed by dissolution in dilute nitric acid. This offered the "best combination of speed, accuracy and precision." In the four methods tried by Daun's group, all but the preferred one ("d") included subsequent development of molybdenum blue for colorimetry, whereas "d" made use of yellow molybdovanadophosphate instead. Daun's preliminary presentation indicated that the great advantage of molybdovanadophosphate over molybdenum blue was the former's lack of sensitivity to the acidity of the medium (12).

*Quantitation of Inorganic Phosphate.* Once the organic material has been digested to orthophosphate, phosphate may be determined as one of two complexes with molybdenum—molybdenum blue or yellow molybdovanadophosphate. To develop the blue color, phosphate is treated, within a specified pH range, with a molybdate solution (usually the ammonium salt) and a reducing agent. Many of the modifications to the Fiske-SubbaRow procedure (3) seek to facilitate the procedure by minimizing the number of solutions required and maximizing the shelf life of the reagents. A good example is the technique of Vaskovsky and coworkers (13), whose combined molybdate-hydrazine reagent is stable for months under refrigeration. All analyses require parallel runs with inorganic phosphate standard solution (usually  $\text{KH}_2\text{PO}_4$ ). One group (14) has used a scintillation counter to quantitate the blue color. The method relies on the quenching of radiation from an external  $^{14}\text{C}$  source. Response is linear over a wide range of phosphorus concentration, from 0 through 0.4 mg P. Table I outlines several reports on phosphorus analysis by molybdenum blue determination. Some serve best for analysis of small amounts of isolated phosphatide, while others may be used for determining trace phosphorus in oil matrices. Some procedures have been adapted for automated analysis. Some involve transfer of the colored complex to an organic phase prior to colorimetry. The table attempts to summarize any information given on sensitivity, accuracy and precision.

The development of molybdovanadophosphate yellow instead of molybdenum blue offers the advantage of room temperature color development, together with less sensitivity to pH and to interfering ions. Recent studies with the yellow complex are outlined in Table II. Note that the automated application was for high concentrations of inorganic phosphate.

A programmable desktop calculator is convenient for repetitive calculations of phosphorus content from colorimetric analysis. A program for a Texas Instruments TI-59 calculator/PC-100C printer is available from the author. This program incorporates linear regression analysis of phosphorus standards.

**TABLE 12-I.**  
**Phosphorus Analysis by Molybdenum Blue Determination**

Digestion	Chromogenes	Sensitivity and repeatability	Reference
HCl/SnCl <sub>2</sub> , 105°/16 hr (A). <sup>a</sup>	Molybdate/ascorbate/ K(SbO) tartrate (A).	CV < 2.5%, 0.5–20 ppm P.	15
3N HNO <sub>3</sub> /0.1 M K <sub>2</sub> B <sub>4</sub> O <sub>7</sub> , then ash (A).	Molybdate/ascorbate (A).	CV = 3% at 0.3 µg P. Not designed for oil.	16, 17
—	Molybdate/ascorbate (A)	CV < 1%. Designed for inorganic P.	18
NaOH/ethanol in Teflon® crucible.	p-Methylaminophenol sulfate/NaHSO <sub>3</sub> / Na <sub>2</sub> SO <sub>3</sub> .	CV = 1% at 60 ppm P. In later study (ref. 7): >> ± 5 ppm error.	9
ZnO, then char, pyrolyze; dissolve in HCl.	Molybdate/hydrazine.	In later study (ref. 7): ± 2 ppm error, but lengthy and cumbersome.	10
H <sub>2</sub> SO <sub>4</sub> /HClO <sub>4</sub> (4/1).	Molybdate/ANSA/ NaHSO <sub>3</sub> /Na <sub>2</sub> SO <sub>3</sub> . All dry-blended in mortar.	—	19
H <sub>2</sub> O <sub>2</sub> /10 N H <sub>2</sub> SO <sub>4</sub>	As above, but no blending. Highly cited method.	—	20
70% HClO <sub>4</sub> , heated.	As above.	—	21
70% HClO <sub>4</sub> at room T.	As above. The original Mo blue method.	—	3
Several equally applicable to plant lipids.	Molybdate/SnCl <sub>2</sub> / hydrazine (A).	CV = 7% at 1.7 µg P.	5, 6, 22
70% HClO <sub>4</sub> H <sub>2</sub> O <sub>2</sub> /H <sub>2</sub> SO <sub>4</sub>	Molybdate/hydrazine. Molybdate/ascorbate. Highly cited method.	CV = 2% at 0.1 µg P. In later study (ref. 24): CV = 1%–2% at 0.9 µg P.	13 23
<i>Variations:</i>			
No digestion.	Molybdate/HCl/ H <sub>2</sub> SO <sub>4</sub> /Hg in chloroform— methanol. Blue color extracted by nonane. Read at 710 nm.	CV = 5% at 10 µg P. No interference by inorganic P.	25
No digestion.	As above, but extracted by chloroform.	Emulsion problems; demonstrated on peanut oil.	26

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**TABLE 12-I (Continued).**  
**Phosphorus Analysis by Molybdenum Blue Determination**

Digestion	Chromogenes	Sensitivity and repeatability	Reference
No digestion.	Molybdate/SnCl <sub>2</sub> .	CV = 0.7% at 1 ppm P.	47
Phosphatides extracted by glacial acetic acid.	Blue color extracted by chloroform. Read at 735 nm.		
HClO <sub>4</sub> /HNO <sub>3</sub>	Molybdate/HCl. Color extracted by n-butyl acetate. Read at 310 nm.	CV = 3%-4% at 0.7-10 µg P.	27
	Similar to above, but tungstate instead of molybdate. Color extracted by n-amyl alcohol. Read at 267 nm.	"Same precision, accuracy, sensitivity as molybdate methods."	28
	Mo blue read by scintillation technique.	Very large range: 0-0.4 mg P.	14
Mg(NO <sub>3</sub> ) <sub>2</sub> pyrolysis.	Phosphomolybdate/ malachite green/ Triton X-100® complex. Read at 650 nm after 5 min at room T.	CV = 2%, 0.1-0.5 µg P.	29
H <sub>2</sub> O <sub>2</sub> /10 N H <sub>2</sub> SO <sub>4</sub>	Similar complex to above. Read at 623 nm.	CV = 1%-2% at 30 ng P.	24
—	Seven dyes studied, incl. malachite green.	Rhodamine B most sensitive and suitable.	30
—	Phosphomolybdate/ methylene blue complex in 0.9 M HNO <sub>3</sub> . Color extracted by methyl isobutyl ketone. Read at 655 nm.	CV < 4%, 0.8-8.0 µg P.	31
	Phosphomolybdate/ ethyl violet complex. Color extracted by methyl isobutyl ketone. Read at 602 nm.	CV 1.3%, 0-0.6 µg P	32

**TABLE 12-II**  
**Phosphorus Analysis as Molybdoavanadophosphate**

Digestion	Chromogenes	Sensitivity and repeatability	Reference
ZnO/H <sub>2</sub> O/O <sub>2</sub> , bomb (up to 0.8 g oil).	Molybdate/ metavanadate. Read at 400 nm.	Later study (ref. 7): ± 4 ppm P error; lower limit 1–2 ppm P; best of the tested methods.	11
4 N HClO <sub>4</sub> , 95 C (A).	As above. Read at 420 nm, (A) Designed for P <sub>2</sub> O <sub>5</sub> of fertilizers.	CV < 1% for 7%–43% P (collaborative study; official method).	33, 34
Cellulose/MgO, ash in muffle furnace.	As above. Read at 400 nm.	CV = 4%–10% at 630 ppm P.	35
MgO, ash in muffle furnace.	As above. Read at 460 nm.	(Official method)	36
<i>Variation:</i>	As above; Differential- pulse voltammetric determination at glassy carbon electrode. Flow injection. Measured at +0.32V (A).	CV < 1% at 10 ppm P. 1 ppm measure- ments are practical.	37

<sup>a</sup>(A) = automated procedure.

Calculator input commands are signalled by appropriate alphanumerics and output is designed in that case for "percent phospholipid." A modification to allow output as "ppm phosphorus" is available from the author on request.

#### Gravimetric

Some official methods are based on precipitation of phosphorus as a complex. An AOAC micromethod (38) involves the gravimetric determination of an ammonium phosphomolybdate, whereas the same precipitate is titrated in the AOCS method for total phosphorus of vegetable lecithins (39). Other AOAC methods measure quinolinium phosphomolybdate [( $C_9H_7N$ )<sub>3</sub> · H<sub>3</sub>PO<sub>4</sub> · 12MoO<sub>3</sub>] gravimetrically (40) or by titration (41).

#### Specific Ion Electrode

Although there is no specific ion electrode for phosphate ion, an indirect method using a fluoride ion electrode may be used to determine phosphate

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over the range 0 to  $2 \times 10^{-4}$  M (0–20 ppm  $\text{PO}_4$   $\pm$  0.5 ppm; 0–7 ppm P). Lanthanum nitrate is added to the digested sample to form very insoluble lanthanum phosphate. Excess lanthanum, still in solution, then is titrated with sodium fluoride. If sulfate is in the sample (as it would be were sulfuric acid used in the digestion step), it is precipitated out with barium ion; lanthanum sulfate is soluble (42). Titration of excess  $\text{Ce}^{4+}$  ion after precipitation of  $\text{Ce}_4(\text{PO}_4)_3$  offers a related approach (43).

### **Ion Chromatography**

Ion chromatographs are high performance liquid chromatography (HPLC) instruments designed to separate and quantitate ionic species at the ppm and even ppb levels. Predigested samples may be passed through a series of ion exchange columns. Eluates then are analyzed by conductivity detection (44). Typical runs require less than 10 min. The technique may be applied to the analysis of trace phosphatides by detection of phosphate ion after digestion. It is also conceivable that phosphatide analysis may be accomplished by ion chromatographic analysis of phosphatide base residues (choline, ethanolamine, serine, etc.) after sample digestion with phospholipase D (or phosphobase residues after phospholipase C).

### **Analysis of Phosphatides without Prior Digestion to Inorganic Phosphorus**

Most of the innovative approaches to phosphatide analysis attempt to avoid the initial digestion steps of the traditional methods. Yoshida et al. have experimented with complexation of phosphatides with transition metals. Complexation with tetrathiocyanatocobaltate resulted in analyses heavily skewed toward phosphatidylcholine (PC) and sensitive only to unsaturated molecular species (45). Complexation with dithiocyanatoiron reagent and subsequent extraction of the complex into 1,2-dichloroethane for spectrophotometric analysis at 470 nm showed linear response to concentrations of all PC in the range 20–240 nmol; the method was sensitive to other phosphatide subclasses, although the response of the acidic phosphatides was often considerably diminished (46). This method would not be suitable for the analysis of soy phosphatides, for example, because a major component—phosphatidylinositol (PI)—gave a response only 23% of PC's. Totani et al. have developed a molybdate-based system that is sensitive to undigested phosphatides. The phosphatides are removed from soy oil by extraction with glacial acetic acid. An aliquot of the acetic acid solution then is treated with a molybdate-stannic chloride preparation and the resulting blue color removed by chloroform extraction for colorimetric determination. Sensitiv-

ity and repeatability are excellent ( $CV = 0.7\%$  at 1 ppm P) (47). Note, however, that chloroform is a potential carcinogen and therefore should be used with caution. Other examples of direct analysis of phosphatides are given in subsequent sections of this chapter.

*Atomic Absorption Spectrometry.* The one instrumental technique that has gained wide acceptance as an alternative to the wet chemical methods is atomic absorption (AA) spectrometry. Direct measurement of traces of phosphorus had been unfeasible in the flame mode due to low sensitivity, although excellent precision and accuracy have been obtained for materials such as fertilizers that contain high amounts of phosphorus (48). In the mid-1970s, however, trace amounts of phosphorus were analyzed successfully using a flameless instrument equipped with a graphite furnace and an electrodeless discharge lamp (EDL). Sensitivity of the signal (213.5/213.6 nm doublet) was enhanced sixfold by incorporation of lanthanum ion, so that phosphorus could be detected down to 10 ng (49). For application to the analysis of trace phosphorus in oils, one must look to the two papers by Prevot and Gente-Jauniaux. In the first paper (50), these workers showed that phosphorus could be analyzed in amounts less than 1 ppm in oils by use of flameless atomic absorption, combined with an EDL. Oil samples in methyl isobutyl ketone were injected directly (no need for predigestion). The second paper (51) gives an improved technique, in which lanthanum is included as its oil-soluble cyclohexylbutyrate salt. Sensitivity to 10 ng phosphorus was established with a coefficient of variation (CV) of 10% at 5 ppm phosphorus. This technique has been applied in other studies to gasoline additives (110 ppm phosphorus) (52), and most interestingly, to whole tissue samples (53). In the latter citation, leaves, bovine liver and oyster tissue were analyzed in amounts under 1 mg. Phosphorus was detected in amounts under 1 microgram with CV's 6% to 10%.

Daun et al., in their study of several methods of phosphorus analysis (7), concluded that oil analysis by atomic absorption spectroscopy could be relied on to give repeatability to  $\pm 3$  ppm ( $CV = 5\%$ ). The authors were hesitant to recommend the technique due to the expense of the instrumentation but praised the technique for its accuracy, precision, sensitivity and rapidity.

*Atomic Emission Spectrometry.* With the exception of one emission technique, namely molecular emission cavity analysis (MECA), emission spectrometry has seen little application for analysis of phosphorus in oils.

MECA instrumentation (54) was designed as an inexpensive supplement to atomic absorption, where those species least sensitive to atomic absorption would be candidates for MECA analysis. Early targets were sulfur and phosphorus, though the developers of the process have concentrated heav-

ily on sulfur. The MECA instrument's small sample container, the cavity, is heated in a hydrogen-rich flame to about 500 C. Phosphorus determinations are done by determining the resulting emission of HPO species at 528 nm. Knowles et al. (55) claimed a CV of 10% for 0 through 50 ppm phosphorus in determining  $\text{PO}_4^{3-}$  and  $\text{P}_2\text{O}_7^{3-}$  ions, AMP, adenosine-5'-triphosphate (ATP) and phytic acid. However, they did not achieve direct fractionation of the different phosphorus species concurrent with the MECA analysis; such fractionation is achievable for different sulfur species (sulfate, sulfite, thiosulfate, etc.). Daun et al. (7) evaluated MECA for the analysis of phosphorus in oils by following the procedure of Rogers and Downey (56) (who reported CV = 10% at 3 ppm) and reported a detection limit of 30 ppm for inorganic phosphorus and two ppm for organic phosphorus. MECA, like atomic absorption, was praised for its rapidity and minimal sample size requirement. Hesitancy was expressed over its cost, however, and MECA was found to have less sensitivity to phosphorus than does atomic absorption. Recently, MECA has been coupled to HPLC for quantitation of organophosphates (57). The authors claimed a sensitivity of 5 ng phosphorus, with a CV of 3% over the range of linear response (10–100 ng). A wheel of 40 cavities collected the column eluate, one drop per cavity. Problems with incomplete burnoff must yet be overcome.

In one study designed to analyze trace phosphorus in water, samples were vaporized to 2800 C in a graphite furnace (as opposed to 500 C in MECA instrumentation), then carried in a nitrogen stream to a flame photometer for measurement of HPO emission. Sensitivity was reported to three ppm, CV 2% to 3% (58). Recently, phosphorus emission was used for detection in an HPLC system. Detection limits were below one ppm, especially for nonionic materials. The system used a dual flame setup—one to reduce or eliminate solvent interferences and the other to bring about HPO emission of the resulting nebulized sample. Flame photometry also has been coupled with gas chromatography (GC). A recent report, for example, describes the analysis of glycerophosphate isomers (silylated) in commercial phospholipids (59).

Plasma emission spectrometers, meanwhile, base their operation on excitation in an electrical arc. Emission analysis of phosphorus is recognized as an AOAC official method (60). A review outlining the differences between two types of plasma emission spectrometers—inductively-coupled plasma (ICP) vs. direct-current plasma—recently was published (61). Other similar reviews cover ICP vs. atomic absorption (62,63). The last reference cites the following detection limits for phosphorus in ppb: a) with atomic absorption—flame, 30,000; graphite furnace, 20. b) with ICP—sequential, 20; simultaneous with other elements, 60. One group claims that phosphorus may be

detected at less than one ppb by ICP as reduced molybdoantimonyl phosphoric acid, by extracting the derivative from water by diisobutylketone (64). An ICP device has been coupled to an HPLC system for analysis of trace amounts of nucleotides. Sensitivity was 750 ng, with a CV of 4.5% at 10 micrograms; phosphorus emission was measured at 213.6 nm (65). Direct analysis of phosphorus in powdered rock samples has been achieved with a sensitivity of 100 ppm (CV = 5%-15%) (66). Kantor et al. have analyzed vegetable oils for phosphorus by (a) ashing a 1-g sample with 0.2 g MgCO<sub>3</sub> and bringing to 200 mg with graphite powder, then (b) by packing 30 mg of this mixture into a hollow graphite electrode and extruding it into an electrical arc. Phosphorus was determined at 255.3 nm relative to magnesium at 273.3 nm. The detection limit was six ppm phosphorus, with a CV of 4.8% at 30-50 ppm (67). More recently, experiments have been reported on the direct aspiration of vegetable oil (in kerosene solution) into an argon plasma arc. Analysis of phosphorus at 213.6 nm was accomplished in less than a minute down to 0.5 ppm (CV = 20%; CV = 5% at four ppm). The same procedure was used to quantitate other trace elements, such as copper, often at the ppb level. Adjustments had to be made continually for drifting of the output signal (68). It seems that interest in the plasma technique will increase as new instruments appear on the market competitive in pricing to atomic absorption spectrometers.

Another technique, that of X-ray Emission Spectrometry (XES), has been used for direct phosphorus analysis of thin-layer chromatography (TLC) spots and column chromatographic eluates. XES/TLC requires calibration curves for each subclass of phospholipids. Lipids were shown to be detectable with linear response from 0.4 to 8 µg P (CV = 5%-10%) (69).

*X-ray Fluorescence Spectrometry.* This technique has been used for rapid multi-element analysis of freeze-dried food samples. CV values of 5% were obtained for phosphorus in orchard leaf samples (1,930 ppm) and in bovine liver (10,700 ppm) (70). The technique requires that samples be mixed with cellulose powder and then pressed into a pellet. Because self-supporting pellets are not feasible for samples whose fat content surpasses 60%, this would preclude analysis of oils for trace phosphorus.

*Neutron Activation Analysis.* Neutron activation analysis certainly cannot yet be considered routine. The technique, which measures gamma radiation of decaying species after neutron bombardment, requires a beta emitter. Commercial services are available that accept samples for such analysis. One report showed that 10 to 30 ng of phosphorus could be determined in this way with a CV of 2% to 3% (71).

*Infrared Analysis.* Recent advances in infrared spectrometry allow the examination of carbonyl absorbances of various lipid species in a mixture and

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subsequent quantitation of the species based on subtle differences in absorbance maxima from species to species. Examination of the absorbance at four different frequencies and consolidation of the data into a matrix of four linear equations allowed the determination of PC in a mixture that also contained triglyceride and sterol ester. Errors ranged from 4% to 30%, but the instrument performed all calculations on-line, thus allowing a rapid analysis (72).

*Chronoamperometric Analysis.* One group has experimented with a dropping mercury electrode for the determination of PC in aqueous methanol or ethanol. The method depends on the adsorption of surfactant-type molecules onto the mercury and the resulting inhibition of the redox process. PC was detected at the 1–8 ppm level of phosphorus, with a CV = 1% (73).

*<sup>31</sup>P Nuclear Magnetic Resonance (NMR) Spectroscopy.* Biochemists and biophysicists have given considerable attention in the last 10 years to <sup>31</sup>P NMR analysis of biological tissue and synthetic membranes. Techniques require the use of instrumentation with Fourier-transform capability, and spectra are accumulated with <sup>1</sup>H-<sup>31</sup>P decoupling. The considerable work of Glonek and his coworkers in analyzing intact animal tissues has appeared in review form (74). Quantitative work using <sup>31</sup>P signals requires optimizing the spectra by assuring uniform spin-lattice relaxation ( $T_1$ ) and by controlling the nuclear Overhauser effect (NOE). London and Feingenson (75) demonstrated that <sup>31</sup>P NMR could be used for quantitation of the phospholipid subclasses of 50 mg of soybean lecithin sonicated in H<sub>2</sub>O/D<sub>2</sub>O. Detrimental peak broadening was controlled by addition of ethylenediaminetetraacetic acid (EDTA) and a detergent (such as potassium cholate). Use of aqueous dispersions allowed pH control, useful for shifting the signal of one subclass away from that of another subclass. Relative ratios for the phosphatides PC:PI: phosphatidylethanolamine (PE): phosphatidic acid (PA) in a half hour experiment were 1.00:0.60:1.06:0.31, respectively, vs. 1.00:0.48:0.95:0.26 by two-dimensional TLC and wet chemical analysis of the subclass spots. The nature of the acyl groups on each subclass did not effect the signal shift. The authors claimed a detection limit of three ppm phosphorus. Absolute quantitation was not reported, although it should have been achievable using another phosphorus species as internal or external standard. A representative spectrum is shown in Figure 1. Gurley and Ritchey (76) were able to analyze phosphorus-containing species (organic phosphates, phosphonates and thiophosphates) in chloroform using <sup>31</sup>P NMR. They, too, optimized conditions for NOE and  $T_1$  and used as a relaxant iron ethylene glycol and shift reagent Gd(fod)<sub>3</sub>. Detection limits (signal-to-noise ratio = 2) for trimethyl phosphate and triethyl phosphate were six ppm and 11 ppm, respectively, in 25-min experiments. Tetraethylammonium phosphate in a precision

capillary tube was used as an external standard. Response factors varied from one phosphate ester to another due to changes in steric bulk and the resulting hindrance to approach by the relaxagent Gd(III).

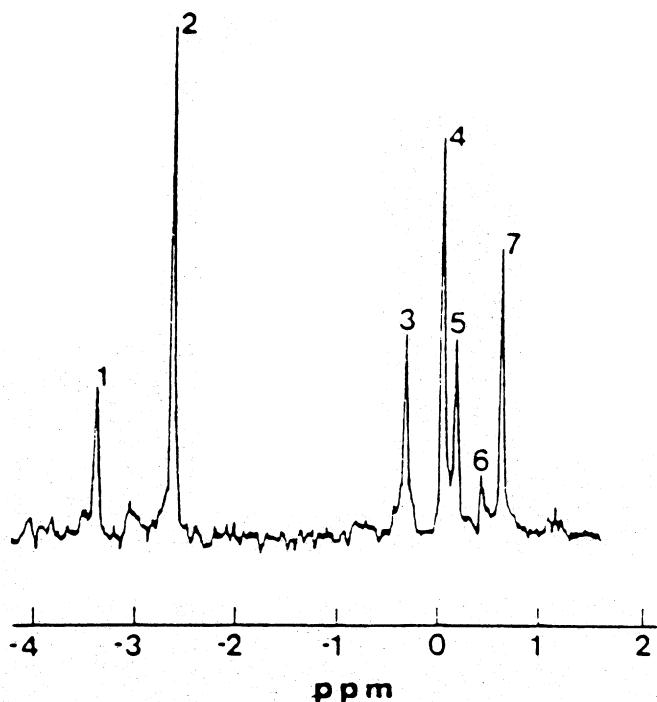


Fig. 12-1.  $^{31}\text{P}$ NMR spectrum of a phospholipid mixture consisting of 5% w/v cholate, 50 mM EDTA, and the following: peak 1, 10 mg of PA; peak 2, 6.6 mg of  $\text{K}_2\text{HPO}_4$ ; peak 3, 8 mg of DPG; peak 4, 13 mg of PE; peak 5, 12.5 mg of PS; peak 6, PI present as an impurity in PS; peak 7, 12.5 mg of dipalmitoyl PC. Total volume 1 mL, pH ~8. 100 transients were collected with an acquisition time of 2 sec per transient, no delay between transients and a filtering time constant of 1 sec (75).

Kasler and Tierney (77) experimented with phosphorus detection in aqueous sulfuric acid, with special attention given to accuracy and repeatability. Triphenylphosphite in chloroform with the relaxagent chromic acetylacetone ("Cr(acac)<sub>3</sub>"), all sealed into a capillary, served as external standard. Quantitation of  $\text{KH}_2\text{PO}_4$  in the 500 ppm range (w/v) with added  $\text{CrCl}_3$  gave a CV value under 2%. Organic compounds first were digested in the NMR tubes with sulfuric and perchloric acids, then quantitated for phosphorus. CV values were similar to the CV for  $\text{KH}_2\text{PO}_4$ .

Although  $^{31}\text{P}$  NMR has become a respected research tool, its use for routine analysis of trace amounts of phosphorus is not yet established. The more complex spectral patterns of lipid mixtures by  $^{13}\text{C}$  or  $^1\text{H}$  NMR preclude routine analysis of trace phosphatide content, though specific lipid subclasses may be analyzed by their characteristic signals. The quaternary ammonium group of PC gives rise to distinct  $^{13}\text{C}$  and  $^1\text{H}$  methyl resonances (78). The  $^1\text{H}$  methyl resonance recently was used to determine the PC content of soy lecithin to the same precision as obtained by HPLC (CV under 7%) (79).

#### **The Phosphatide/Phosphorus Ratio**

All the methods discussed above lead to a quantitation of phosphorus. This is in essence a molar quantitation of phosphatide, because the main phosphatides-PE, PI, PC—bear one phosphorus atom per molecule. Lipid biochemists often use an arbitrary factor of 25 to estimate the weight of phosphatide from the weight of phosphorus. A mixed acyl PC that bears one palmitoyl group and one linoleoyl group would have a molecular weight of  $776/(776 \text{ g PC/mol PC}) (1 \text{ mol PC/g-atom P}) (1 \text{ g-atom P}/31.0 \text{ g P}) = (25.0 \text{ g PC/g P})$ , hence, the factor 25. Obviously the problem becomes complex when PC bears a variety of fatty acyl groups, more complex when the phosphatide mixture is more than just PC, and in the case of "vegetable lecithin," even more complex when the product contains other materials mixed with phosphatides (materials such as neutral lipids, glycolipids, saccharides and amino acids). This requires empirically determining the ratio. The results of several empirical determinations appear in Table III.

#### **Enzymatic Analysis**

Quantitation of total phosphatide content by enzymatically assisted reactions is yet a goal for future research, although one group (85) has reported such a feat. Phospholipase C (EC 3.1.4.3) is used to convert phosphatides to 1,2-diglycerides. Lipoprotein lipase (EC 3.1.1.3) then liberates glycerol, which may be detected colorimetrically in the presence of  $\text{NAD}^+$ , glycerol dehydrogenase (EC 1.1.1.6), and phenazine methosulfate nitro blue tetrazolium dye. Corrections must be made for interference by triglycerides in the lipid to be analyzed. This interference has caused another research group to label this procedure as unsatisfactory (86).

Most enzymatically-assisted analyses of phosphatides have been specific for choline-containing lipids PC, lysophosphatidylcholine (LPC) and sphin-

**TABLE 12-III**  
**Conversion Factors: The Phosphatide/Phosphorus Ratio**

Oil source	Acetone insolubles from		
	Freshly obtained lecithin	Raw oil	Deoiled lecithin
Soy	32.07 ± 1.26 <sup>a</sup>	31.69 ± 1.38 <sup>b</sup> 30 <sup>c</sup> 32.7 <sup>d</sup> 32.1 ± 1.4 <sup>e</sup>	30.7 <sup>a</sup>
Rape	31.75 ± 1.14 <sup>a</sup>		
Peanut	24.4 <sup>f</sup>		

<sup>a</sup>Ref. 80.<sup>b</sup>Ref. 81.<sup>c</sup>Ref. 10.<sup>d</sup>Ref. 82.<sup>e</sup>Ref. 83.<sup>f</sup>Ref. 84, determined from total lipid extract of seeds.

gomyelin (SPH). Choline typically is liberated by phospholipase D (EC 3.1.4.4) (86–94), although this also may be achieved with phospholipase C (EC 3.1.4.3) together with phosphatase (EC 3.1.3.1) (95). Choline then may be used to generate hydrogen peroxide in the presence of choline oxidase (EC 1.1.3.17). Quantitation of hydrogen peroxide may be done by dye coupling in the presence of peroxidase (EC 1.11.1.7) (86,88,90–93). A commercial kit for choline analysis by dye coupling (phospholipase D/choline oxidase/peroxidase/4-aminoantipyrine) is available from Nippon Shoji Company, Osaka, Japan (PL kit K and autoanalyzers PL kit K “f”) and is based on the method of Takayama et al. (88). A reaction scheme for this process is shown in Figure 2. Choline quantitation also may be achieved by voltammetric means (89), by a choline dehydrogenase (EC 1.1.99.1) system (87,95) or by a choline kinase (EC 2.7.1.32) system (94,96). A commercial kit based on a choline kinase system (96) is available from Boehringer Mannheim Biochemicals, Indianapolis, IN 46250, and Mannheim, Federal Republic of Germany (Lecithin Kit 529 362). Details of all of these enzymatic methods are outlined in Table IV.

Another method requires phosphorus in inorganic form at the outset. A purine nucleoside phosphorylase (EC 2.4.2.1)/xanthine oxidase (EC 1.2.3.2)/uricase (EC 1.7.3.3) system is used to generate hydrogen peroxide, which is measured as quinoneimine dye after a coupling reaction catalyzed by peroxidase. The four-enzyme system requires 15 min at 37°C and may be used over a range of 0 through 1.6 ppm P (97). The initial reaction—the conversion of orthophosphate and isosine to ribose-1-phosphate and hypoxanthine—is used in a related method that utilizes HPLC for quantitation;

### Plant Phospholipid Analysis

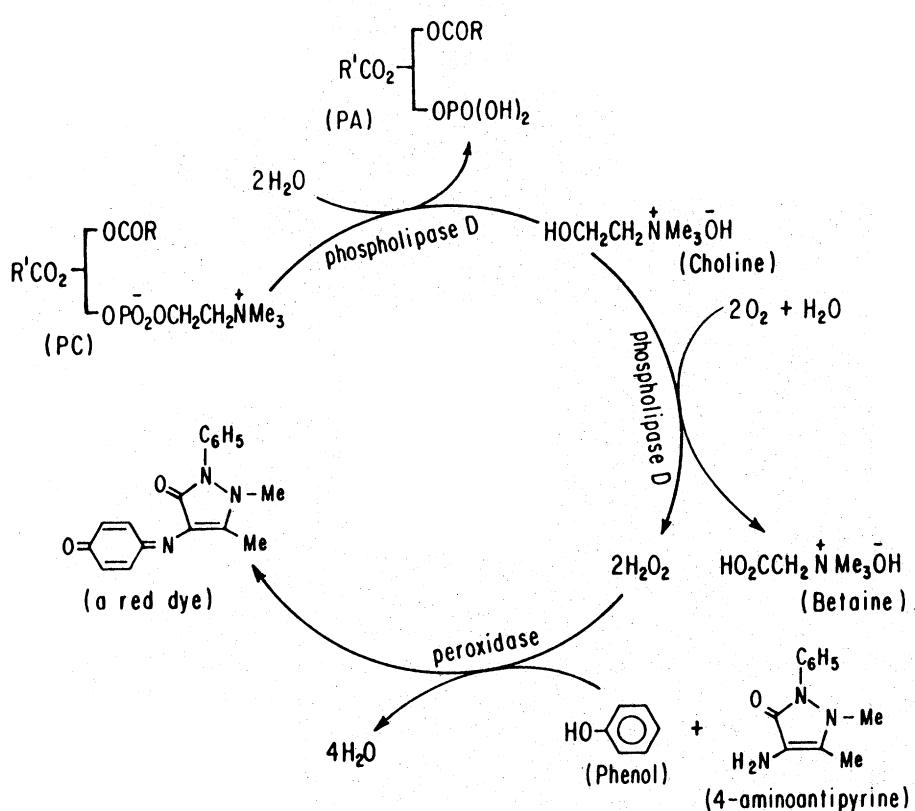


Fig. 12-2. Reaction scheme for enzymatic determination of PC (88).

hypoxanthine is separated from inosine and related to orthophosphate concentration. Linearity is claimed through 30 ppm phosphorus ( $CV = 10\%$ ) and the detection limit is 0.75 ppm (97a).

The future may see applications of enzyme technology to specific non-choline-containing phospholipids. Researchers already have identified PI-specific phospholipases C that show no detectable attack on any other phospholipids (98).

### Subclass Analysis

Because many fine review papers and monographs have been published on the isolation, separation and identification of lipid classes, this review will

TABLE 12-IV  
Enzymatic Analysis of Phosphatides (choline-species only, unless specified)

Substrate	Choline liberation	Choline reaction	Quantitation	Remarks	Reference
Serum (Commercial enzyme kit available; cf text).	Phospholipase D. liberates 2 H <sub>2</sub> O <sub>2</sub> .	Choline oxidase	Peroxidase, dye coupling, UV/VIS.	CV .48% at 76 ppm P. CV .45% at 116 ppm P. Range 0-400 ppm P. 97% "recovery" at 14 ppm P.	88
All phosphatides of serum.	Phospholipase C liberates DG; Lipoprotein lipase then liberates glycerol.	Glycerol dehydrogenase + NAD <sup>+</sup> + dye.	—	Comparable to choline oxidase in sensitivity, but cf. ref. 86 below.	85
Bile (procedure of ref. 88).	Phospholipase D.	Choline oxidase.	Peroxidase, dye coupling.	CV 3%-6% at 15-79 ppm P. Also, found ref. 85 (above) unsatisfactory, due to LPC, DG, TG, in sample.	86
Bile.	Phospholipase D.	Choline oxidase.	Peroxidase, dye coupling.	Linearity through 300 ppm P.	93
Bile (procedure of ref. 83).	Phospholipase D.	Choline oxidase.	Peroxidase, dye coupling.	Linearity 60-300 ppm P. CV .67% at 60 ppm P.	92
PC of amniotic fluid.	Phospholipase D from cabbage.	Choline oxidase.	Peroxidase, dye coupling.	CV 87% at 1 ppm (16:0) <sub>2</sub> PC	90
PC of amniotic fluid.	Phospholipase D from Streptomyces	Choline oxidase.	Peroxidase, dye coupling.	CV .58% at 4 ppm SPH chromofuscus.	91

will focus only on novel developments most relevant to plant phosphatides.

#### **Extraction**

Extraction of phosphatides from tissue most often has been done with chloroform-methanol systems. Two of the most widely cited articles are the extraction methods of Folch et al. (99) and Bligh and Dyer (100). These methods are relied on for efficient extraction of total lipid and minimal contamination from nonlipid materials. Many define "lipid" as tissue material that is isolated by a Folch or Bligh-Dyer method. Numerous variations have appeared subsequently, including one that shows that chloroform may be replaced by dichloromethane (101). Extraction by a dry column method, using dichloromethane and methanol, eliminates much of the tedium associated with the methods cited above and allows separation of lipids into neutral and polar fractions simultaneously with extraction (102). Although the method was developed for meat products, it also has been used on peanuts (103). Another group has shown that lipids may be extracted efficiently using a hexane-isopropanol system (104).

Two studies have dealt with the problem of isolation of lipid that is tightly associated with protein. One group utilizes proteolytic enzymes to liberate this lipid and to increase the lipid yield in a Folch extraction (105). The other group, mainly concerned with the elimination of non-lipid impurities in a lipid extract, reverses the usual extraction-then-wash sequence by pretreating the tissue (immature soybeans) with hot dilute acetic acid prior to chloroform-methanol extraction. This reversal removes the normal nonlipid contamination of the lipid extract and denatures hydrolytic enzymes (106). Users may encounter difficult emulsion problems using this technique. It should be stressed that the enzyme phospholipase D is abundant in premature seeds and, if not denatured prior to extraction, leads to rapid trans-phosphatidylation upon contact with methanol (107). It is very probable that many an unknown phosphatide, identified in charts and figures as the ubiquitous PX, is actually the artifact phosphatidylmethanol. A recent study has compared the application of several extraction procedures to soy products (108). The study showed that a Folch-type method was as efficient as two day-long Soxhlet extractions (one with choroform-methanol, the other with benzene-ethanol) and more efficient than a short extraction with hexane-ethanol. A similar study has been reported on such legume seeds as kidney beans, field beans, lentils, chick peas and peas; chloroform-methanol was more efficient than diethyl ether, although acid hydrolysis prior to extraction gave the highest yields of fatty acids (109). Yields of PI suffer from

*Plant Phospholipid Analysis*

TABLE 12-IV (Continued)  
Enzymatic Analysis of Phosphatides (choline-species only, unless specified)

Substrate	Choline liberation	Choline reaction	Quantitation	Remarks	Reference
"Lecithins" of serum.	Phospholipase D.	Choline dehydrogenase gives betaine aldehyde, and simultaneously: (ED 1.2.1.8) gives NADH (UV).	Reduced dye (OR NAD <sup>+</sup> + betaine aldehyde dehydrogenase (ED 1.2.1.8) gives NADH (UV)).	—	87
PC, SPH of serum	Phospholipase C liberates phosphocholine. Then phosphatase liberates choline.	Choline dehydrogenase gives betaine aldehyde, and simultaneously: Denature, then choline kinase + ATP → ADP.	Reduced dye (OR NAD <sup>+</sup> + betaine aldehyde dehydrogenase (ED 1.2.1.8) gives NADH (UV)). ADP quantitation by pyruvate kinase and lactate dehydrogenase.	CV < 1%	95
a. PC in foods b. PC of amniotic fluid	As above				96
PC of serum	Immobilized phospholipase D.	Immobilized choline oxidase.	H <sub>2</sub> O <sub>2</sub> measured voltammetrically.	CV 5% at 120 ppm P. range 60–140 ppm P.	89
—	Phospholipase D.	Choline kinase + ATP → ADP.	Pyruvate + NADH (340 nm) → NAD <sup>+</sup> ADP + phosphoenolpyruvate → pyruvate.	Presented as method of phospholipase D quantification; not yet used for PC/LPC.	94

Sephadex LH-20 gel allowed good recovery not only of phosphatide fractions but also for sunflower and rapeseed lipids (116) and for soy (117). Use of the advantages of using gel chromatography were demonstrated by Biacs et al. reported in detail for soy lecithin.

ether/ether, then C with ether, leaving behind d. Experimental results were and (d) nonlipids were adsorbed. Fraction b was eluted with petroleum ether, while (b) triglyceride and sterol esters, (c) sterols and free fatty acids, (a) the phosphatides (and hydrocarbons) passed through the column until solution with petroleum ether. By virtue of micelle formation in this solvent, solvated in petroleum ether and passed through a deactivated silica gel column with silica gel. Commercial lecithin oil was dissolved in petroleum ether and passed through a deactivated silica gel column (115) to occur on silica gel. An unusual phenomenon has been reported (115) to occur on silica gel.

*Column Chromatography (Non-HPLC).* An unusual phenomenon has been reported (115) to occur on silica gel.

*2-Oleoyl-PC, etc.).*

Perhaps, have been in the use of HPLC to separate the phosphatides by sub-class (PC, PE, etc.) and by molecular species (diplamitoyl-PC, L-palmitoyl-phosphatides, etc.), have been in the use of HPLC to separate the phosphatides by separation of vegetable phosphatides, are discussed below; the most significant work of Nelson (114). Recent development, especially as they pertain to the counter-current technique of phosphatide fractionation, see the work of Nelson (114).

For a general review and critique of phosphatide fractionation, see the counter-current technique with petroleum ether and aqueous ethanol (113). From nonpolar lipids may be done using a series of separatory funnels and a some type of chromatography. A tedious bulk separation of polar lipids to some who use a traditional extraction method usually resort directly (102), those who use a traditional extraction method concentrate the dry column method of extraction does yield a phosphatide concentrate neutral lipid requires a way to concentrate the desired fraction. Although neutral lipid requires a way to concentrate the desired fraction. Although

### Chromatography

The researcher who seeks the extraction of total lipids from an oilseed made in an article by Kinsella et al. (112). Relative lipid data depend on proper technique, a point well made in an article by Kinsella et al. (112).

Lipid losses during aqueous washing could be minimized by clean-up of a crude extract on acid-washed alumina. The researcher who seeks the extraction of total lipids from an oilseed must follow procedures directly at odds with the procedures applied for commercial isolation of crude oils. The isolation of total lipids implies the maximum concentration of phosphatide yielded, whereas the commercial isolation of phosphatide yields a phosphatide concentrate which follows standard extraction techniques for the isolation of vegetable lipids. Lipid losses during aqueous washing could be minimized by clean-up of a crude extract on acid-washed alumina.

### Plant Phospholipid Analysis

demonstrate improved efficiency of separation of phospholipid components, lipids was reviewed in 1982 (123). There are several recent reports that *High-Performance Liquid Chromatography (HPLC)*, Progress in HPLC of the column without adsorption.

bisphosphatidylglycerol (DPG), then PI phosphatidylserine (PS), then PA, then PI (which included some sulfated), then diacylglycerol (DAG), then PI phosphatidylserine (PS), then PA, then PI (which included some sulfated); method could be used to isolate preparative amounts of the following lipids: of neomyctin's role as a weak anion exchanger. He demonstrated that the (including PI), Palmer (123) then expanded the method by taking advantage a method to isolate polyphosphoinositides free of other phospholipids neomyctin's affinity for molecules with multiple phosphogroups, developed ones to obtain is the use of immobilized neomyctin. Schachet (122), noting A novel approach to isolate subfractions that are usually the most difficult if present, with the next seven. All other phosphatides do not elute (121).

draws off neutral lipid. PC is eluted with the next ten liters and sphingomyelin,

(decreasing in acetone content as elution progresses). The first three liters is begun (30 mL/min) with a binary solvent system of acetone/methanol

of 300 g of adsorbent has been charged with 10 g crude phosphatide, elution

achieved on a column of hydroxyapatite ( $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ ). After a column

A large-scale isolation of PC from crude egg or soy phosphatide has been elutes the phosphatides (120).

Another group has been able to use a dry column chromatographic method to isolate an intact phosphatide fraction. Up to 800 mg of total lipid is applied to five g silica gel. Elution of the column with isopropanol draws off neutral lipid, sterols and free fatty acid. Then ammoniated methanol is applied to the alumina column with ethanol and the silica gel column with

eluted the alumina column with ethanol and the silica gel column with

as he also had done in his lipid extraction procedure (104). Instead, he

allowed the recovery of cleaner PC. Radiin avoided the use of toxic solvents,

included small amounts of sphingomyelin loading

for the second. A fraction close to two g was almost all PC, though it

pressure for the first column and then 60 psi (by chromatographic pump)

procedure (alumina, then silica gel) by Radiin (119), using slight helium

gram amounts of egg yolk lecithin were fractionated by a two-column

len to 70% PC, an unusually high percentage of total phospholipid. Ten

(which contained a trace of cerebroside). However, 4.2 out of six g is equiva-

fractionated on silica gel to give 4.2 g pure PC, 0.2 g pure LPC, and 1.2 g PE

tions but also of glycolipids (including cerebroside, sterol glycosides).

optimized separations on silica by use of a gradient elution with hexane-brain and liver lipids and numerous model lipids were used in a study that subclasses have been reported on silica and ion exchange columns. Rat subclASSES have been reported on silica and ion exchange columns. Rat For analytical purposes, good separations of phosphatides into their from the first run was re-injected separately for further purification (128).

Separation in a two-step procedure, where each of two fractions isolated PC, and sphingomyelin fractions. A 26-g sample of the same mixed lipid was baseline separation of five egg yolk phospholipid into neutral lipid, PE, gel columns were used with chloroform-methanol-water systems to obtain separation also was seen (127). In the other study, radially compressed silica no evidence for molecular species for accounting for sphingomyelin or PS. Some evidence for molecular species for PE, PI, lysophosphatidylethanolamine (LPE) and PC, in that order, with chloroform-methanol gradient elution. Baseline separations were achieved yolk phospholipid were separated on a 10-m silicic acid column, using a scale isolation of phospholipid subclasses. In one study, ten grams of egg

Two publications demonstrate how HPLC may be used for preparative analysis has not yet been reported.

which uses a woven fibrous quartz belt. Successful utilization in phosphatide Traceor Instruments has recently introduced its model 945 LC/FID detector, with a perforated band (cf. below) have not yet been adopted commercially. wire detectors were available commercially; Privet and coworkers' results Flame ionization detection (FID) suffered a poor reputation when moving the need for aqueous components for HPLC separation of phosphatides. has not become widespread, probably because of its lack of sensitivity and chromatographic groups to various lipid subclasses. Interred detection (126) standard for lipids. Others have experimented with the attachment of the 205 nm wavelength region as the closest to what may be labelled as regarding detection seem to have settled for UV detection in users by its efficiency.

Phosphatides by HPLC (125). Such a systematic approach often surprises its has been shown to be a good approach for optimizing the separation of need not be a hit-or-miss one. The use of a simple optimization procedure for this or her particular case. The process of finding a proper solvent system and adjust the HPLC system by experiment to optimize the separation Therefore, the researcher must view any recommendation as a guideline and column efficiency, to allow successful separations on first attempt. There are too many uncontrollable variables, such as column temperature, lipid area—the use of microbore columns (124). Regarding separation efficiency, most reported techniques are extremely difficult to reproduce; other reports that attack the problem of detection, and finally a new development in HPLC for which there are as yet no reported applications in the lipid area—*the use of microbore columns (124)*. Regarding separation

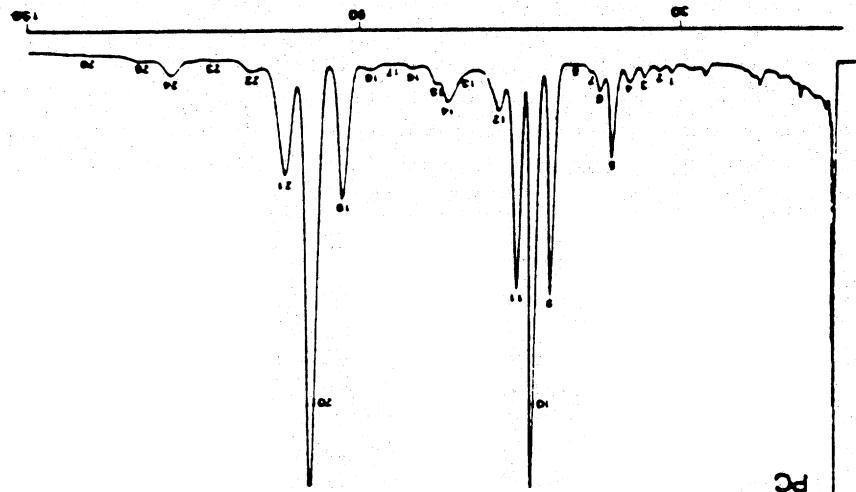
Others have resorted to separations on ion exchange type columns. Col- umns of amino functionalities bonded to silica (*u*Bondapak-NH<sub>2</sub>, Waters Associates, Milford, MA) were used with gradient elution. One group used a chloroform-methanol-water system on a synthetic mixture of phospholipids with flame ionization detection (135), while another used a hexane-isopro- panol-methanol-water system on egg yolk phospholipids with detection at 206 nm (136). A cation exchange column of silica covariance bonded to benzene sulfonate functionality (PXS 10/25 SCX, Whatman, Clifton, NJ) served to separate rabbit myocardial lipids; isocratic and gradient runs with acetoniitrile-methanol-water all allowed good separations, except for PE/PS. There is a trend now to accomplish the separation of intact, as opposed to derivatized, molecular species of phospholipid subclasses. Compaction and Purdy (138) focused on sharpening the peak of diacyl-PC (reversed phase column, aqueous methanol). Elevated column temperatures and addition of either acid (sulfuric, perchloric, but especially phosphoric or diethylphos- phoric) or ion-pairing agents (triethylammonium sulfate) greatly improved efficiency, presumably by suppressing adsorption and thus emphasizing partition on the reversed phase column. Earlier, another group successfully separated a synthetic mixture of saturated PCs using methanol-water-

isopropanol-water-sulfuric acid. Detection was at 205 nm. Such an acid system is not suitable for plasma-ogen-containing animal lipids (129). Another good separation on silica was achieved using acetone:methanol:85% phosphoric acid, again using rat tissues and standards, with detection at 203 nm (130). Yet another group used acetonitrile-methanol-water and detection at 210 nm to analyze small amounts of PC in chocolate (131); the same group later (79) compared this method to an NMR method (choline methyl) absorption (to achieve the same analysis). Excellent results recently were reported by Patten et al., who separated rat liver lipids using an isocratic system of hexane-isopropanol-25 mM phosphate buffer-ethanol-acetic acid (132). Detection was at 205 nm. Soy phosphatides were separated on a silica column using hexane/isopropanol/acetate buffer (or acetic acid) systems and detection at 206 nm (123). The same column (Lichrosorb Si 60) was used by another group to achieve a more impulsive separation; the use of toluene or benzene in the latter separation precluded UV monitoring, however, and relied instead on a flow-through radiometric detector (with

a short capillary column (2F-2330) by GC (141,142). Another derivatization approach was used by Hsieh et al. (143) with the derivatized lipid (egg yolk PC) separated into molecular species by reversed

Myher and Kuskis, meanwhile, have been able to remove the polar headgroup of phospholipids by hydrolysis in the presence of phospholipase C, then separate the resulting diglycerides as their silyl ether derivatives on

Figs. 1-2-3. HPLC separation of molecular species of PC (from rat liver) (132). Conditions: C18 reverse phase column ( $4.6 \times 250$  mm Ultrasphere ODS, Altex Scientific, Inc., Berkeley, CA), Load, 220  $\mu$ g. Eluting solvent, 20 Ml methanol/water/acetonitrile (90.5:7.2:5). Flow rate, 2.0 mL/min. Detection, 205 nm. Each numbered peak is from one molecular species (two for peaks 2, 4, 5, 14, 16, 22) (e.g., peak 9 = 16.0-22.6 PC, peak 10 = 16.0-20.4 PC, peak 11 = 16.0-18.2 PC, peak 12 = 18.1-18.2 PC).



chloroform (no adsorption suppression) on reversed phase columns; results with egg yolk PC were not so effective. Refractive index detection was used (139). Better results were seen in a later study using egg yolk PC and a methanol-1 M phosphate buffer system and UV detection at 205 nm (140). To date, the best reported results are those of Patrón et al. (132), who obtained sharply defined separations (Fig. 3) of the molecular species of PC, obtained from the rat liver using a methanol-acetonitrile-water system that contained choline chloride (20 mM). Similarly good separations were obtained by this group for PS, using methanol-25 mM KH<sub>2</sub>PO<sub>4</sub>-acetonitrile-acetic acid that also contained choline chloride (30 mM). Patrón's group used UV detection at 205 nm.

Plant Phospholipid Analysis

six reasons why TLC is likely to remain the most used separation procedure: Centrury of Thin-Layer Chromatography—an Interim Report” (156), cites “Thin-Layer Chromatography (TLC). Egon Stahl, in his review of “A Quarter subclass (154,155).

Central data allows a reconstruction of the fatty acid profile of each lipid species by gas (153). Using a computer to assemble and interpret the mass single ion monitoring in the chemical ionization mode using methane as reagent gas (152), or into a mass spectrometer for identification by for quantitation (151,152), or reduce the hydrocarbons. The hydrocarbons are swept into the FID to remove solvent, then into a reactor where cracking and hydrogenolysis mode, the column eluate is carried on the band through an evaporator steel band as a transport system between an HPLC column and both a flame ionization detector and a chemical ionization mass spectrometer. In the FID private and coworkers have reported their work with a perforated stainless

two peaks have the same degree of unsaturation (150).

detector, one may monitor at 195 nm and at 215 nm to determine whether mize between saturated and unsaturated lipids; using a variable wavelength Recently a call has been made for monitoring at 215 nm as the best compromise between phospholipids in hexane-isopropanol-water systems (149). the separation of phospholipids to add a fluorescent chromophore (147).

an HPLC column. UV detection has been studied in detail at 206 nm with (88) to quantitate choline-containing phosphatides as the lipids elute from group (148) has even used the enzymatic methodology of Takayama et al. esters of phosphatidic acid (143) and to silyl ethers of diglycerides (141). One Already mentioned was the conversion of phosphatides to the dimethyl chloride) to add a fluorescent chromophore (147).

nm (146), or with dansyl chloride (L-dimethylamino-naphthalene-5-sulfonate biphenylcarbonyl chloride to yield derivatives that may be detected at 280 and serine-containing phospholipids have been reacted either with 4-and 230 nm in dioxane systems (145). The amino groups of ethanolamine cerebrosides, allows detection at 280 nm in ethyl acetate-containing solvents and 230 nm in dioxane systems (145). The availability of hydroxy or amine groups on the lipid depending on the amidation of amino groups with chromophoric acylation agents, detection of lipid subclasses has been achieved by esterification of hydroxy groups or amidation of amino groups with chromophoric acylation agents, their absorptivity never has produced a universal chromophore. Success in detection.

William N. Marmer



The weak element of TLC always has been quantitation. Phosphatides contain a marker by virtue of their phosphorus atom and therefore may be

light (174).

hexatriene, which reveals lipid as violet spots under long wavelength UV blue spots under short wavelength UV light (173). The other is 1,6-diphenyl-the lipid spots. One is N-phenyl-L-naphthylamine, which reveals lipid as nondesctructive reagents are recommended for visualization (172). Two the use of an acid-fuchsin-maranyl nitrate reagent for removal from guard against hazards buildup of perchloric acid. Another group praises bright green spots on a light orange background (171). Care must be taken to reveal may be treated with a molybdate reagent and Malachite green to reveal by spraying with perchloric acid and then heating the plate. The plate then hand, phospholipid may be converted on the plate to inorganic phosphorus on a yellow background; inorganic phosphorus is inert (170). On the other and is sensitive to 40 ng phosphorus. Phospholipids are seen as blue spots and is used as TLC visualization reagents (13), some require heating for color development while some also are unstable on the TLC plate. The use may be used as TLC visualization reagents (13), some require heating for although molybdate reagents commonly used for phosphorus analysis

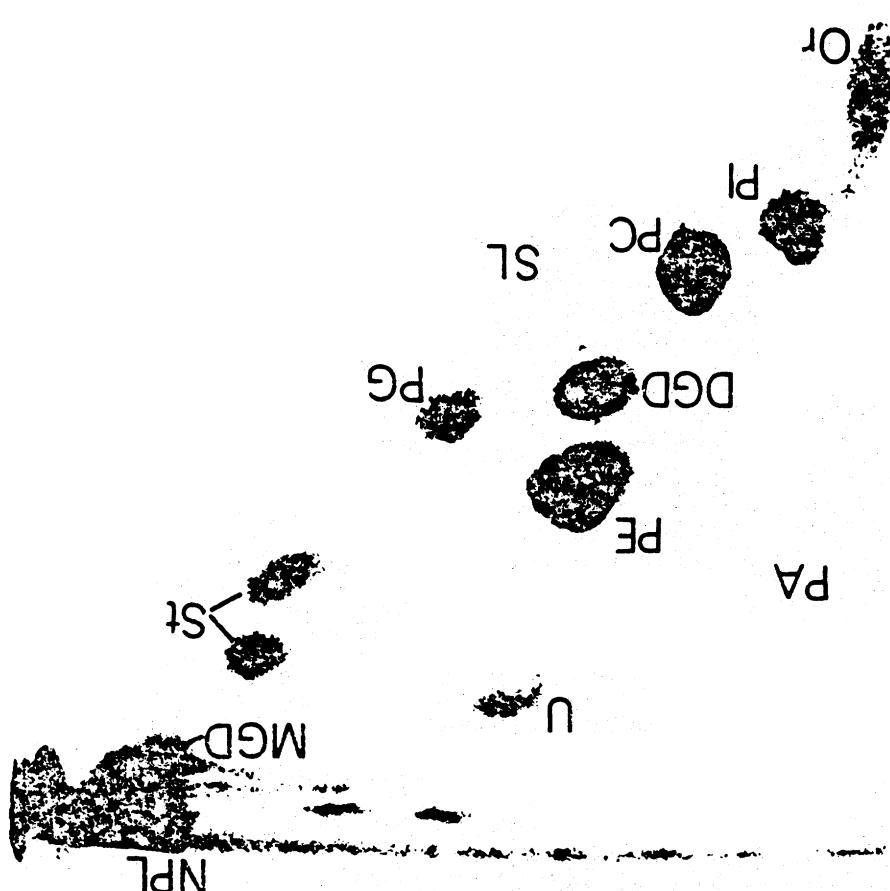
need for centrifugation prior to colorimetric analysis (169).

TLC spots may be scraped and digested for phosphorus analysis without any alumina over silica gel is the solubility of the adsorbent in strong acid. The tides on basic alumina (with 10%  $\text{CaSO}_4$ ). The chief advantage of using Finally, an older reference teaches the method of separating the phosphorus/20% silver nitrate using a chloroform/methanol/water (65:25:4) system. trifluoroacetamide derivatives (168). Separations were achieved on silica applied to the resolutions of the molecular species of PE and of PS as their ammonia (125:75:6:2) for development (167). Aggregation TLC has been on boric acid-imregnated silica plates using chloroform/methanol/water/excellent separation of DEAE-silica gel to column chromatography (166). solution of applications of DEAE-silica gel to column chromatography (166). Rapid and efficient separation of phosphatides was achieved under-

phorylcholine (GPC), glycerophosphate and betaine (165). Separated inorganic phosphate, choline, choline phosphate, glycerophosphate, (5:2:1:2:2), followed by methanol/water/ammonia (130:55:8:4:4). This method was an extension of precursors has been run on silica gel using chloroform/methanol/water/pyridine/ammonia (130:55:8:4:4). This method was an extension on (diethylamine) ethyl-modified silica gel using chloroform/methanol/water/ammonia (130:55:8:4:4). This method was an extension of applications not the phosphatides but their polar degradation

internal standard. Peak areas are translated into weights and then related to followed by GC analysis of the resulting methyl esters in the presence of an similar approach uses the fatty acids as a marker. Transmethylation is or X-ray emission methodology discussed at the beginning of this chapter. A analyzed by scraping the TLC spots and proceeding with the wet chemical

per text; U = unknown; St = sterols; NPL = nonpolar lipids. Other abbreviations as glycerol; Or = origin; SL = sulfolipid; MGD, DGD = mono-, digalactosyl diacyl-imature soybean cotyledons (steam-killed prior to extraction) (107). Conditons as per text. Fig. 12.4. Two-dimensional TLC separation of polar lipids from 100 mg fresh weight



*Plant Phospholipid Analysis*

This dipping technique led to the staining of the background of the plate. DCF dye was used in acetone solution into which TLC plates were dipped. With concentration, but each subclass had its own slope (183). Later, the same 550 nm dipping excitation at 365 nm. Lipid subclass responses were linear to detect 10-50  $\mu$ g of lipid with a CV = 5%-15% by scanning emitted light at example of dye treatment, 2,7-dichlorofluorescein (DCF) allowed one group fluorescent dyes or by heat treatment in the presence of a reagent. In one aqueous. Fluorescence has been induced by treatment of the spots either with liquids. Others have favored fluorometric densitometry over the charting technique. (182).

A novel approach to quantitation of charred spots involves not densitometric scanning of spots on plates, but instead the quenching produced when the scraped charred adsorbent is suspended in a scintillation counting gel that contains a radioactive source (radium-226 external standard pellet) (181). Lipids could be determined rapidly in a range of 10-1000  $\mu$ g with a CV under 5%. The authors claimed that variation in response due to degree of unsaturation was minimal. Response was nonlinear with respect to concentration, and therefore required the preparation of standard curves. Later saturation, and the more rapid the preparation of standard curves. Later others have favored fluorometric densitometry over the charting technique. (182).

CV values generally under 10% for nanogram amounts of phosphatides separated by HPTLC were due to scanning mode to achieve effects of varying unsaturation on charting intensity (179). Phosphatides required hydrogenation of the lipids prior to TLC to cancel the phytol spot technique in the transmission mode on red blood cell phosphatide, used acetate, in phosphoric acid (178). An earlier study, which used the intensity may be doubled by using copper sulfate, instead of the common and linearity was experienced over the range 0.1-10  $\mu$ g lipid (177). Charting background noise. Maximum absorbance occurred at 350 nm wavelength referred over transmission measurements because the latter produced higher resolution and by different responses from lipid subclasses to subclasses.

At present analyses of densitometric scanning of charred TLC spots showed that improvements in repeatability by use of the newer "flying spot" technique over the older "split scanning" were minimal [CV = 10.3%, 11.2%, respectively, for 7  $\mu$ g (18:1)<sup>2</sup>PC spots], although the flying spot technique was best for irregularly shaped spots. Repeatability measurements were performed over irregularly shaped spots. Repeatability by shape and intensity. Furthermore, variations are introduced by the degree of unsaturation and by different responses from lipid subclasses to subclasses.

Alternatively, one may visualize the TLC spots and quantitate by some type of scanning. Densitometric scanning, however, has its problems, espe-

cially with regard to variations in charting or staining caused by spot size,

shape and intensity. Furthermore, variations are introduced by the degree

(FID). Development takes place not on TLC plates but instead on thin film the principles of TLC with quantitation by flame ionization detection. Thinchromatography (Rod TLC), meanwhile, is a method that aims to conclude.

This method and the ammonium bicarbonate one (190), these authors under 5%. Fluorescence required polymers such as lipid spot both for quantitation could be achieved with as little as 20 pmol of PI with a CV nm. Fluorescence of lipid spots was detected above 500 nm upon excitation at 360 nm. Solution (acetone/conc. perchloric acid/conc. hydrochloric acid, 250:10:6). Plates at 110 C for 30 min after dipping them in a dilute perchloric acid solution (acetone/conc. perchloric acid/conc. hydrochloric acid, 250:10:6). The best sensitivity was seen by a group that heated its developed HPTLC though each phosphatide subclass required its own calibration curve (189).

plates. Excellent repeatability (CV = 2%) was seen down to 1 mol of lipid (188). This technique was used in a later study on silica gel 60 HPTLC (188). The reagent in the vapor state allowed uniform treatment of the plate was linear in the range of 2–100  $\mu$ g upon excitation at 380 nm. Application saturation. PC response (455 nm emission) at the square root of peak area Schiff base forms with malonaldihyde released during oxidation of polyunsaturated fatty acids in a sealed tank with solid ammonium bicarbonate. Apparently a heating the developed silica gel-alumina (2:1, no binder) plate to 110–150 C. Good sensitivity was achieved by inducing the lipids to fluoresce by fluorescence methods above.

As has been mentioned already, with detection in HPLC, chromophoric groups may be attached to amino groups of such lipids as PE and PS. In one example (187), lipids were reacted with fluorodinitrobenzene and then subjected to TLC. The resulting derivatives could be seen as yellow spots against a white background. However, densitometric response was linear in the range 0.02–0.4  $\mu$ mol of derivative, considerably less sensitive than the range of 60–1000 ng (186).

An alternative is 6-p-tolidino-2-naphthalenesulfonic acid, which is more sensitive than ANS, and—for cholesterol at least (no experiments were performed with phosphatides)—is removable from the lipid and allows separation of the researcher wish to recover the intact lipid from the removal (185). This dye, however, had been criticized (173) for its difficulty of removal a nearly dark nonfluorescent background. PC could be detected to 200 ng (ANS) because, unlike DCF or rhodamines, it produced fluorescence spots on another team recommended ammonium 8-anilino-1-naphthalenesulfonate measured at 504 nm with excitation at 312 nm wavelength (184).

Lipid concentration then was related to the diminution of fluorescence. Dipalmitoyl-PC then could be quantitated in the range of 15 ng–1  $\mu$ g with a CV under 6% by relating the response to a standard curve. Response was measured at 504 nm with excitation at 312 nm wavelength (184).

The utility of thinchromatography as a qualitative and "semiquantitative" method has been confirmed, but its value for quantitative determinations is controversial. Operational factors that can lead to improved results include proper humidity control of unspotted rods, very careful spotting technique, FID, adequate equilibration with solvent vapors, good evaporation of solvent after development, proper scanning speed and adequate rod cleaning. Nevertheless, imadequacies still persist. There is variability from rod to rod and in the orientation of the rod within the flame. The range of sensitivity is approximately 1–30  $\mu$ g lipid, thus precluding the detection of components under 3% of a mixture (unless a large spot is burned off selectively) and more than 10% had been experienced in routine operations (196). Another group also claimed a CV of 5% but noted a great difference in response for different classes of lipids (197). The advantage of using internal standards has been cited. L-Octadecanol has been used as an internal standard in a study of polar lipid separation, the introduction of LPG as an internal standard improved precision from 12%–20% ( $20\text{--}5 \mu\text{g}$ ) to 7% (199). Other groups, reporting quantitative errors in excess of 10%, have hesitated to recommend the use of thinchromatography in quantitative applications (200–202). The last reference points out significant problems with responses factors, reportng quantitative errors in excess of 10% have hesitated to report to another, this group also noted that responses factors varied with the position of the spot on the rod. This was a problem because  $R_f$  values changed with repeated use of the same rod.

ethers. Saito's group has examined molecular species of fetal lung PC as the other groups have examined phospholipids as their diglyceride silyl EI-MS.

The acetylated products of egg yolk PC (210) and of total phospholipids from bacteria (211) allowed molecular species identification by "conventional" HPLC examination of bacterial PE, PG, and DPG (209). Earlier work with undervent conversion to the acetylated diglyceride, GC/CI-MS with NH<sub>3</sub> MS of derivatized lipids. For example, one group studied PC after the lipid molecules, although the system is incapable of handling mixed constituents. Monitored conversion of phospholipid subclasses have been identified by the profile, although the most rapid means of establishing a fatty acid monitoring (154) offers the most rapid means by specific ion HPLC detection. The group's identification of fatty acids by section on another instrument (HPLC, GC), has been discussed in the section on The work of Privett's group, in which a CI-MS instrument is coupled to of these soft-ion systems (208).

MS (EI-MS) (207). Another review focuses on the electronics and mechanics advantages of these methods over the more conventional electron-impact ionization (CI-MS) and field ionization for lipid analysis demonstrates the ionization (206). A review of FD-MS and other "soft-ion" MS methods [chemical can be used to determine the carbohydrate sequence of these complex lipids ions (203-205). Recent work with glycosphingolipids has shown how FD-MS failure in the analysis of PS and difficulties with contamination by metal included problems with  $(M + 1)^+$  intensity with phosphatidylglycerol (PG), natural mixtures including egg yolk and amniotic fluid PCs. Difficulties minimal fragmentation. This group with some success experiments with temperature to achieve the highest intensity of mass ions ( $M + 1$ )<sup>+</sup> and pattern of 50 model phospholipids and optimized the FD-MS fragmentation phytides. Wood and coworkers have studied the FD-MS fragmentation of undervatized relatively nonvolatile molecules, including several phos- MS in the field desorption mode (FD-MS) has enabled the examination ing is its inability to provide good quantitation of components of a mixture. proven to be a remarkable tool for structural elucidation, a major shortcoming is its use for rapid determination of fatty acid profiles. While MS has determined the distribution of molecular species. MS methodology also has led to methods for examining intact and derivatized phospholipids to have led to interesting developments in mass spectrometry (MS).

Mass Spectrometry, Interesting developments in mass spectrometry (MS) time that HPLC is suitable for routine analysis of phosphatides. point out that comparisons to TLC are unfair, that analogies to TLC are only superficial. When accuracy and precision are important, researchers will probably find their time better spent using TLC techniques until such have led to carrying out routine TLC. Proponents of the Iatroscan have led to methods for examining intact and derivatized phospholipids to determine the distribution of molecular species. MS methodology also has been used for rapid determination of fatty acid profiles. While MS has

Advances in GC and data handling technologies have made fatty acid profiling considerably more sophisticated than was the case a decade ago. With the increasing detail that is seen by use of capillary columns comes the need to handle more and more data. Esterification processes have not changed greatly since they were reviewed critically in 1975 (215). My coworkers and I have found one particular transmethylation reagent — KOH/methanol — to be highly satisfactory when free fatty acid content is negligible. The method (216) is protective of enol ether groups of the plasma lipids commonly found in the polar lipid fractions of animal, but not plant, tissues. However, it is incapable of transmethylating the fatty acyl moieties of ceramide lipids. When free fatty acids are present, they form soaps which then must be esterified with an acidic system such as one that uses  $\text{BF}_3/\text{methanol}$  (217). A recently reported alternative process uses tetramethylammonium hydroxide and methanol in ether. As transesterifica-

tion proceeds, the glycerol byproduct forms a lower layer and retains any free fatty acids of the original lipid as their tetramethylammonium soaps. Because these soaps pyrolyze in a GC injector to give methyl esters, injection of the glycerol layer leads to a profile of the free fatty acid fraction. On the other hand, injection of the ether layer leads to a profile of the transmethylated glycerides. Alternatively, the two layers may be made mis-

obtainable (218). Analogs to the reaction with tetramethylammonium hydrox-

**Gas Chromatography (GC).** Methodology using GC has been mentioned throughout this review, in the notes on molecular species separation (in the discussion of HPLC) (41), in notes on TLC quantitation (175,176), in conjunction with mass spectral analysis (154,209,212,213) and with flame photometric detection of phosphorus (59).

A technique that has not yet been applied to phosphatides is the combination of TLC with MS. A recent report (214) described the direct introduction of phenols, sterols, nucleosides and derivatized amines and amino acids together with their TLC adsorbent (polyamide) into the ion source of the spectrometer. The method should be applicable to the analysis of phosphatides, although general use with lipids is restricted by the nature of the bases, although some success has been reported with silica gel and alumina failed, the report noted, because vaporization in the source always led to decomposition.

trimethylsilyl ethers, and noted that no isomerization from the 1,2-diglycidide form to the 1,3-diglycidide form occurred (212). Slighit isomerization was noted by those who examined the acetylated derivatives discussed above. Later work by Saito's group showed the use of *tert*-butylidimethylsilyl ether derivatives in molecular species analyses by GC-MS (213).

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well (219). Times, is assigned  $R_{RT} = 1$ . In our work, the palmitoleate peak served conveniently chosen within a bunch of peaks that have similar retention times initially reported actual retention times. A commonly occurring peak, that substitutes the more reproducible relative retention times ( $R_{RT}$ ) for manual assignments are facilitated by generation of a modified report editing) (224). My coworkers and I have found that in repetitive profiling, identities manually (219,223) or semiautomatically (i.e., with manual duplicate. This makes it mandatory that raw profiles be assigned their peak dublicate. This problem usually arises because retention times just are not so repeatable, especially when several peaks elute with similar retention times. This problem usually arises because retention times is often that automated peak identification by retention time windows is often by preheating the empty syringe needed in the injection for three-five seconds prior to depresssing the plunger. Finally, in assembling the voluminous data minimized by using larger sample volumes (e.g., 2  $\mu\text{L}$  instead of 1  $\mu\text{L}$ ) and volatile ones. A recent study (222) implies that discrimation may be bottling components leave the syringe needle in lower proportions than the we should be aware of the phenomenon of "discrimination", i.e., that high because most of us researchers are tied to sample introduction by syringe, tion of one ester over another (221).

Because most of us researchers are tied to sample introduction by syringe, polar fractionation could be avoided with regard to transesterification. First, chloroform should be mentioned as a solvent during this process, because the ethanol present in chloroform as a stabilizer will lead to the formation of ethyl ester artifacts (220). Second, a solution of methyl esters should not be subjected to a stream of gas for concentration. Lower homologs will evaporate preferentially to leave behind a solution altered in its relative concentration.

We also have found that considerably greater detail in fatty acid profiling interfere with the subsequent chromatography (218a). methanol is instantaneous, and the  $\beta$ -product dimethyl sulfide does not

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## Appendix

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